The Effects of Potassium, 5-Hydroxytryptamine, Adrenocorticotrophin and Angiotensin II on the Concentration of Adenosine 3':5'-Cyclic Monophosphate in Suspensions of Dispersed Rat Adrenal Zona Glomerulosa and Zona Fasciculata Cells

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(Received 8 January 1974)

Dispersed rat adrenal cells prepared from both the capsule and the decapsulated gland were used to investigate the effects on cyclic AMP accumulation of known stimuli of steroidogenesis [ACTH (adrenocorticotrophin), angiotensin II, K^+ ions and 5-hydroxytryptamine]. Since glomerulosa-cell preparations from capsular strippings are normally contaminated with a proportion of fasciculata cells, cells purified by fractionation on a bovine serum albumin gradient were also used. The results showed that: (1) ACTH and angiotensin II stimulated cyclic AMP accumulation in both fractionated and unfractionated zona fasciculata cells; (2) 5-hydroxytryptamine and an increased extracellular K^+ concentration (from 3.6 to 8.4 mM) had no effect on cyclic AMP concentrations in fasciculata cell preparations; (3) the addition of ACTH, angiotensin II, 5-hydroxytryptamine or K^+ to the incubation medium resulted in increased cyclic AMP concentrations in unpurified zona glomerulosa cell preparations; (4) fractionation and hence the virtual elimination of fasciculata contamination, did not affect the response to 5-hydroxytryptamine and increased K^+ concentration. However, the responses to ACTH and angiotensin II were markedly lowered but not abolished. These results strongly suggest a link between cyclic AMP production and steroidogenesis in the zone of the adrenal gland that specifically secretes aldosterone. All four agents used stimulated both steroid output and cyclic AMP accumulation. However, at certain doses of 5-hydroxytryptamine, K^+ and angiotensin II the significant increases in corticosterone output were not accompanied by measurable increases in cyclic AMP accumulation.

Cyclic AMP has been widely recognized as a ubiquitous regulatory agent which acts as an intracellular mediator of the action of a number of polypeptide hormones and biogenic amines. Studies on the role of cyclic AMP in steroidogenesis in the whole adrenal cortex have revealed that the action of ACTH (adrenocorticotrophic hormone) is probably mediated by alterations in the intracellular concentrations of cyclic AMP (Grahame-Smith *et al.*, 1967; Taunton *et al.*, 1969; Lefkovitz *et al.*, 1970; Carchman *et al.*, 1971; Mackie *et al.*, 1972; Beall & Sayers, 1972; Kitabchi & Sharma, 1971; Birmingham *et al.*, 1960; Sayers *et al.*, 1971; Ferguson, 1963; Karaboyas & Koritz, 1965). However, few attempts have been made to distinguish between the effects of ACTH and other stimuli on the various cell types in the adrenal gland.

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The outer zone of the adrenal, the zona glomerulosa, functions independently from the inner zones, the fasciculata and reticularis, and is the unique site of aldosterone production. Studies *in vivo* have demonstrated that several stimuli (i.e. increased K^+ concentration, angiotensin II and ACTH) act on the adrenal cortex to increase the secretion of aldosterone and its precursor corticosterone. Studies *in vitro* have shown that in addition to these stimuli, 5-hydroxytryptamine (serotonin) and cyclic AMP also stimulate aldosterone production (Haning *et al.*, 1970; Kaplan, 1965; Müller, 1970). However, despite intensive research in the last decade, controversy remains as to the relative importance of angiotensin II, ACTH and K^+ in the control of aldosterone secretion. Considerable interest has been expressed in the suggestion that changes in intracellular K^+ concentrations could be the common denominator in the actions of all known stimuli of aldosterone secretion (Baumber *et al.*, 1971).

The involvement of the adenylate cyclase-cyclic AMP system in the control of steroidogenesis in the zona glomerulosa is largely unknown. To clarify the relationship of cyclic AMP to steroidogenesis, particularly in the zona glomerulosa, we have investigated the effect of known stimuli of steroid biosynthesis on cyclic AMP concentrations in isolated adrenal cell preparations. Corticosterone measurements were performed in parallel. In view of the uncertainty of the effects of stimuli on the conversion of corticosterone into aldosterone we have considered it reasonable to study corticosterone output only. In practice, in all situations studied aldosterone and corticosterone outputs by the zona glomerulosa have been correlated.

Dispersed cell preparations have certain advantages over tissue slices; in particular, target sites should be readily accessible to stimuli; moreover, cells may be fractionated and different types purified. Such purification has been especially important in this study, since cells prepared from adrenal capsular strippings are normally contaminated with a proportion of fasciculata cells. Therefore it is difficult to interpret unequivocally observed effects of ACTH, angiotensin II or cyclic AMP concentrations. For these reasons we have, at various stages of this study, used zona glomerulosa and zona fasciculata cells purified by unit gravity sedimentation in an attempt to identify the effects of such agents on each cell type.

The study reported here forms part of an investigation into the regulations of aldosterone biosynthesis (see J. F. Tait *et al.*, 1972, 1974; S. A. S. Tait *et al.*, 1972). A preliminary report of some of this work has been published (Albano *et al.*, 1973a, 1974a).

Experimental

Materials

[^3H]Adenosine 3':5'-cyclic monophosphate (25 Ci/mmol), which was from The Radiochemical Centre, Amersham, Bucks, U.K., was evaporated to dryness and resuspended in assay buffer before each assay to eliminate radioactivity not associated with cyclic AMP (Brown *et al.*, 1972a; Albano *et al.*, 1974b). Cyclic AMP and deoxyribonuclease I were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Cyclic nucleotide phosphodiesterase was from Boehringer Corp. (London) Ltd., London W.5, U.K. Tris-HCl buffer, 0.05 M, pH 7.4, (assay buffer) contained mercaptoethanol (6 mM) and theophylline (8 mM). Scintillation fluid used for cyclic AMP assay was prepared by dissolving 12 g of BBOT [2,5-bis-(5-*t*-butylbenzoxazol-2-yl)-thiophen] (Ciba Ltd., Duxford, Cambridge, U.K.) and 160 g of naphthalene in 1200 ml of toluene and 800 ml of 2-methoxyethanol. [1,2- ^3H]Corticosterone

(48 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.) and [4- ^{14}C]corticosterone (56.7 Ci/mmol) was from The Radiochemical Centre. Cyclohexane (Spectroquality, Merck, Anderman and Co., East Moseley, Surrey, U.K.) was used without further purification. Methylene chloride was purified by the method of Saffran & Schally (1955) and stored at -20°C . The scintillation fluid used in the corticosterone radioimmunoassay was 300 ml of Triton X-100 + 40 ml of Permafluor (Packard Ltd., Reading, Berks., U.K.) made up to 1 litre with toluene. ACTH (corticotrophin) was ACTHAR (Armour Pharmaceuticals Co. Ltd., Eastbourne, Sussex, U.K.). 5-Hydroxytryptamine creatinine sulphate was from Koch-Light Laboratories Ltd., Colnbrook, Berks., U.K. Collagenase (CLS batch no. 1BF) was a product of Worthington Biochemical Corp., U.S.A. and obtained from Cambrian Chemicals Ltd., Croydon, U.K. Bovine serum albumin for the incubation media and for the bovine serum albumin gradient was Pentex fraction V (Miles Laboratories Ltd., Stoke Poges, Bucks., U.K.). The bovine serum albumin used as an admixture with the charcoal was from Stayne Laboratories, High Wycombe, Bucks., U.K. All other solvents and reagents were A.R. grade from BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

Preparation of cell suspensions. The adrenals from female Sprague-Dawley rats were dissected as previously described (Tait *et al.*, 1974) into capsular and decapsulated glands. Capsular cells were prepared by the method of S. A. S. Tait *et al.* (1972) and the cells from the decapsulated glands by the method of Haning *et al.* (1970). Briefly, the required tissue fractions were incubated in polytetrafluoroethylene beakers in a freshly prepared solution of collagenase (2 mg/ml) and deoxyribonuclease (0.05 mg/ml) in a medium comprising a modified Krebs-Ringer bicarbonate buffer (Krebs & Henseleit, 1932) ($[\text{K}^+] = 3.79 \text{ mM}$), and 0.2% glucose and containing 4% (w/v) bovine serum albumin. (This buffer will be referred to as KRBGA medium.)

The cells were dispersed by repeated pipetting at 25 min and 50 min after which time the cells were washed (Haning *et al.*, 1970) and suspended in KRBGA medium. Cell counts with an improved Neubauer haemocytometer revealed a usual yield of approx. 1×10^5 – 2×10^5 cells/adrenal.

Fractionation of capsular adrenal cells. Purified zona glomerulosa cells were prepared by using the technique of gravity sedimentation through a bovine serum albumin gradient. The theoretical and practical aspects of this technique have been described by Tait *et al.* (1974). In brief, a linear

gradient of 1.5–2.7% bovine serum albumin in Krebs–Ringer bicarbonate buffer containing glucose was formed in a glass vessel. Then 20ml of 1.25% bovine serum albumin in the same medium was added to the top of the gradient followed by 20ml of 1.0% bovine serum albumin in Krebs–Ringer bicarbonate buffer containing the cells and finally by 20ml of 0.75% bovine serum albumin in Krebs–Ringer bicarbonate buffer. The cells from the capsular strippings from the equivalent of 80 glands were sedimented for 2.5h. After sedimentation, the contents were collected in 15–18ml fractions and samples were taken for analysis by Coulter counter, phase contrast and electron microscopy. The fractions containing the zona glomerulosa cells were taken for incubation to measure cyclic AMP and corticosterone production. Purified zona fasciculata cells were prepared in a similar manner except that approx. 40 decapsulated glands were used.

Cell incubation. Incubations were prepared by adding 0.45ml of the cell suspension (equivalent to the cells from one rat) to 0.5ml of KRBGA medium and 0.05ml of acid saline (0.9% NaCl, acidified to pH 4 with HCl). The angiotensin, when added, was dissolved in the 0.5ml of KRBGA medium, whereas the ACTH or 5-hydroxytryptamine were added in the 0.05ml of acid saline. Alterations in K^+ concentration were obtained by changing the concentration of both K^+ and Na^+ in the 0.5ml of KRBGA medium. Iso-osmoticity was maintained, the Na^+ concentration being changed by a maximum of 5%. All incubations were continued for 2h under $O_2 + CO_2$ (95:5) at 37°C in a metabolic shaker. In all experiments duplicate beakers were prepared, one

being taken for corticosterone determination and the other for cyclic AMP determination.

Corticosterone assay. At the end of the incubation the incubates were diluted with water and extracted as described previously (S. A. S. Tait *et al.*, 1967; 1972). Corticosterone was assayed by radioimmunoassay (S. A. S. Tait *et al.*, 1972) by using antibodies raised against corticosterone–21-hemisuccinate–albumin conjugate developed in sheep (Haning *et al.*, 1970).

Cyclic AMP assay. At the end of the 2h incubation the entire contents of each incubation beaker were mixed with 2ml of ethanol, shaken on a Vortex mixer and cooled to –20°C. After 16h the extracts were warmed to 4°C and centrifuged at 1250g for 15min. A portion (2ml) of each supernatant was evaporated to dryness. The dried extracts were resuspended in 200ml of 0.05M-Tris–HCl (pH 7.4) assay buffer. Portions (10µl and/or 50µl) of this extract were then assayed for cyclic AMP by a saturation assay (Brown *et al.*, 1971; 1972a). In some experiments an ion-exchange column-chromatography step was interpolated. The dried ethanolic extracts were resuspended in water and chromatographed on Dowex AG 50W (X4), cyclic [3H]AMP being used as recovery indicator. The eluted cyclic AMP was evaporated to dryness, resuspended in the Tris–HCl assay buffer and assayed for cyclic AMP. We have maintained our normal procedure of assaying the samples against standards set up to include an equivalent amount of a reagent blank extract (Brown *et al.*, 1971; Albano *et al.*, 1973b). The latter was prepared from an equivalent amount of KRBGA medium at the corresponding K^+ concentration

Table 1. *Effect of phosphodiesterase treatment and sample dilution on cyclic AMP assay results*

For details see the text.

Sample	Treatment	Cyclic AMP (pmol/ incubation tube)
Zona glomerulosa cells	Incubated for 2h with 3.6mM- K^+	0.33
	+ phosphodiesterase	0.02*
	Incubated for 2h with 0.1 mM-5-hydroxytryptamine	0.64
	+ phosphodiesterase	0.02*
	Incubated for 2h with 400 µg of angiotensin/ml	0.58
	+ phosphodiesterase	0.02*
	Incubated for 2h with 800 µg of angiotensin/ml	0.81
	+ phosphodiesterase	0.02*
		(pmol/10 ⁶ cells)
	Incubated for 2h with 0.1 mM-5-hydroxytryptamine	6.66
Zona fasciculata cells	Diluted 1:2 with reagent blank	6.86
	Diluted 1:4 with reagent blank	6.16
	Incubated for 2h with 3.6mM- K^+	15.1
	Diluted 1:2 with reagent blank	13.7
	Diluted 1:4 with reagent blank	15.2

* Within the detection limit of the assay.

Table 2. *Cyclic AMP concentrations in suspensions of zona fasciculata cells*

Cells were incubated for 2 h at 37°C in KRBGA medium under O₂+CO₂ (95:5), other details are given in the Experimental section.

Additions	Cyclic AMP (pmol/10 ⁶ cells)					
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5(a)	Expt. 5(b)*
Incubated control (3.6mM-K ⁺)	10.0	4.1	7.4	11.5	15.3	9.9
K ⁺ (8.4mM)	9.6	4.4	7.9	11.7	—	—
5-Hydroxytryptamine (0.1 mM)	10.4	—	8.6	—	—	—
ACTH (0.01 i.u./ml)	59.8	40.4	—	28.0	122.8	82.4
Angiotensin (100 µg/ml)	—	—	—	—	82.19	42.4
Angiotensin (400 µg/ml)	—	—	—	—	483.2	793.3
Angiotensin (800 µg/ml)	—	—	—	—	578.7	904.4
Angiotensin (1600 µg/ml)	—	—	—	—	660.8	1239.7

* Fractionated cells.

Fig. 1. *Effect of angiotensin II on corticosterone and cyclic AMP output by fractionated zona fasciculata cells*

The cells were incubated with various concentrations of [5-valine]angiotensin II for 2 h. Cyclic AMP (b) and corticosterone (a) were determined on duplicate samples.

including ACTH, 5-hydroxytryptamine or angiotensin as appropriate. The measurement of cyclic AMP concentrations after phosphodiesterase treatment did not differ significantly from zero (Table 1) when compared with the appropriate reagent

blank-response curve (Albano *et al.*, 1974b). Samples diluted in the Tris-HCl assay buffer supplemented with the reagent extract produced the expected results (Table 1), but samples diluted in assay buffer alone predictably did not.

Although ethanolic extracts of the KRBGA medium exerted a non-specific effect on the assay, extracts of KRBGA medium containing different concentrations of K⁺ (between 2mM and 13mM) resulted in identical response curves. 5-Hydroxytryptamine in the KRBGA medium also had no additional effect. However, extracts of KRBGA medium containing angiotensin II did cause some interference in the assay. This effect was diminished after ion-exchange chromatography; therefore extracts of samples containing angiotensin were subjected as a routine to chromatography and subsequently compared with standards containing reagents that had been similarly treated. Certain other extracts were also chromatographed in the same way.

Results

Cell incubations

(1) *Cells prepared from decapsulated glands.* The treatment of decapsulated rat adrenal glands with collagenase resulted in suspensions comprising mainly zona fasciculata cells together with cells of the zona reticularis. Only ACTH and angiotensin induced increased concentration of cyclic AMP (Table 2); both increased K⁺ concentrations (from 3.6 to 8.4mM) and 0.1mM-5-hydroxytryptamine were ineffective in raising total cyclic AMP concentrations in these cell suspensions (Table 2). The responses in terms of corticosterone output were

Fig. 2. Cyclic AMP concentrations in suspensions of non-purified zona glomerulosa cells

The effect of increased concentrations of K⁺ [8.4 mM (▨)] as compared with 3.6 mM (□) and 5-hydroxytryptamine [0.1 mM (■)] on cyclic AMP concentrations in seven separate experiments is shown.

Table 3. Effect of various doses of 5-hydroxytryptamine on cyclic AMP and corticosterone output by non-purified zona glomerulosa cells

For incubation details see Table 2.

Additions	Cyclic AMP (pmol/h per 10 ⁶ cells)	Corticosterone (ng/h per 10 ⁶ cells)
Control (3.6 mM-K ⁺)	4.09	68.00
10 nM-5-Hydroxytryptamine	4.01	160.24
0.1 μM-5-Hydroxytryptamine	7.36	139.26
1 μM-5-Hydroxytryptamine	8.69	158.91
0.1 mM-5-Hydroxytryptamine	6.25	175.36

Fig. 1. The output of corticosterone and cyclic AMP from cells incubated in KRBGA medium at K⁺ concentration of 3.6 mM served as the control throughout these studies.

(2) Cells prepared from adrenal capsules. Cells prepared from adrenal capsular strippings by collagenase treatment comprised mainly zona glomerulosa cell types, but with contamination of up to 10% zona fasciculata cells, although this usually amounted to about 5%.

Fig. 2 shows the results of seven separate experiments in which the cells were incubated with 5-hydroxytryptamine (0.1 mM) or with K⁺ at increased concentration (8.4 mM as compared with 3.6 mM), these concentrations being maximal with respect to the corticosterone output of similar cell preparations (Haning *et al.*, 1970; S. A. S. Tait *et al.*, 1972). Both stimuli caused significant but variable increments in total cyclic AMP concentration.

The effect of various concentrations of K⁺ on incubations of unpurified capsular cells is shown in Fig. 3. The pattern of the corticosterone response observed is essentially similar to that previously reported (S. A. S. Tait *et al.*, 1972; Haning *et al.*, 1970). However, in contradistinction to the raised corticosterone output between 3.6 mM- and 5.9 mM-K⁺, no significant increase in cyclic AMP concentrations was observed. In addition, at high (13 mM) K⁺ concentration, cyclic AMP concentrations remained raised, although the corticosterone output was lower than at 8.4 mM-K⁺. At low K⁺ concentrations (2 mM) cyclic AMP concentrations are higher than at 3.6 mM-K⁺, a response which likewise differs from that of corticosterone. Nevertheless, there was a consistent increase in cyclic AMP concentrations at 8.4 mM- and 13 mM-K⁺ as compared with the control (3.6 mM-K⁺).

The effect of various doses of 5-hydroxytryptamine on both corticosterone output and cyclic AMP accumulation is shown in Table 3. Maximum cyclic AMP concentrations were found at a dose of 1 μM; however, corticosterone output was maximal with

Fig. 3. Effect of different concentrations of K⁺ on cyclic AMP (a) and corticosterone (b) output by non-purified zona glomerulosa cells

The concentrations of K⁺ used were 2 mM, 3.6 mM, 5.9 mM, 8.4 mM and 13 mM. Results for three separate experiments [(i), (ii) and (iii)] were shown. For other details see the text.

essentially similar. Zona fasciculata cells purified by unit gravity sedimentation were likewise responsive to angiotensin II, the pattern of the response being essentially similar to that of unpurified cells prepared from the decapsulated gland (Fig. 1). The dose-response curves for both cyclic AMP and corticosterone production of purified fasciculata cells in response to angiotensin II are shown in

Table 4. *Cyclic AMP concentrations in suspensions of non-purified zona glomerulosa cells*

For incubation see Table 2.						
Cyclic AMP (pmol/10 ⁶ cells)						
Control	ACTH			Angiotensin II		
	5m-i.u./ml	10m-i.u./ml	40m-i.u./ml	100 µg/ml	400 µg/ml	800 µg/ml
5.98	—	47.68	—	—	—	—
4.09	—	15.61	—	4.09	—	—
7.38	22.15	26.99	36.35	—	—	—
9.72	—	—	—	8.95	11.02	21.15
14.72	—	—	—	20.91	36.88	53.17
4.58	—	—	—	3.73	7.83	12.52

Fig. 4. *Cyclic AMP concentrations suspensions of purified zona glomerulosa cells*

Cells were purified as described in the Experimental section. This Figure records the results of stimulation with an elevated K⁺ concentration [8.4mM (▩)] and with 5-hydroxytryptamine [0.1mM (▨)] in three separate experiments; □, control.

10nM at which no statistically significant change in cyclic AMP concentration could be observed.

ACTH at 10m-i.u., a dose that is maximal in terms of corticosterone output, caused a significant increase (approx. threefold) in the concentration of cyclic AMP (Table 4) in suspensions of cells prepared from the capsular strippings ('unfractionated zona glomerulosa cells'). In one experiment a lower dose, 5m-i.u./ml, also caused a significant cyclic AMP response; this dose was also maximally effective on steroid production. In this same experiment a dose of 40m-i.u./ml resulted in an even greater cyclic AMP response (approx. fourfold). Similarly, angiotensin II stimulation resulted in increased cyclic AMP concentrations at a dose of 400 µg, and a greater increase (2–4-fold) at 800 µg of angiotensin II. In only one of the three experiments was the lower dose (100 µg) of angiotensin II effective in increasing cyclic AMP accumulation (Table 4).

(3) *Purified zona glomerulosa cells.* The possibility that these effects arose from the contaminating zona fasciculata cells could not be excluded, since both

ACTH and angiotensin II cause large increases in cyclic AMP concentrations in the fasciculata cell suspensions. Therefore zona glomerulosa cell preparations after unit gravity sedimentation, containing less than 0.5% fasciculata contamination as judged by phase-contrast and electron microscopy, were used. All incubations of these fractionated (purified) cells were performed in parallel with incubations of non-purified cells, the purified cells being prepared from the same batch of cells in each case. The cyclic AMP concentration in the control incubations of purified cells was 5.26 ± 1.41 (s.e.m. $n = 8$) pmol/10⁶ cells as compared with 6.47 ± 1.00 ($n = 12$) in the non-purified cells. This slight decrease in cyclic AMP concentration after fractionation is consistent with the observation that the basal concentration of cyclic AMP in fasciculata cell suspensions is higher (9.72 ± 1.54 pmol/10⁶ cells, $n = 6$) than in glomerulosa cell suspensions.

Both 5-hydroxytryptamine (0.1mM) and an increased K⁺ concentration (8.4mM) caused similar increases in cyclic AMP concentrations in incubation of these purified zona glomerulosa cells (Fig. 4) as in the unpurified cells. The effect of ACTH and angiotensin II on purified zona glomerulosa cells is shown in Table 5 and the dose-response curves with angiotensin are shown in Fig. 5. Increases in both corticosterone and cyclic AMP accumulation were still apparent after fractionation, but were significantly less than before fractionation; the responses to both stimuli after fractionation were essentially similar to those with 5-hydroxytryptamine and increased K⁺ concentration. The effect of all four stimuli on cyclic AMP concentrations in both fractionated and unfractionated glomerulosa cells is summarized in Fig. 6. It is clear that the elimination of contamination by fasciculata cells does not affect the responses to 5-hydroxytryptamine or increased K⁺, but markedly affects the response to ACTH and angiotensin II. Nevertheless, both ACTH and angiotensin II cause increased cyclic AMP accumulation even in incubations of glomerulosa

Table 5. Cyclic AMP concentrations in suspensions of purified zona glomerulosa cells

For incubation details see Table 2.

	Cyclic AMP (pmol/10 ⁶ cells)					
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6
Incubated control (3.6mM-K ⁺)	2.80	2.77	6.99	7.13	13.96	3.04
ACTH (0.01 i.u./ml)	7.37	5.80	17.50	—	—	—
Angiotensin 100 µg/ml	—	3.20	—	6.57	14.15	2.74
Angiotensin 400 µg/ml	—	—	—	10.34	24.53	—
Angiotensin 800 µg/ml	—	—	—	10.78	21.89	5.41

Fig. 5. Effect of various doses of angiotensin II on cyclic AMP (b) and corticosterone (a) output by fractionated zona glomerulosa cells

[5-valine]Angiotensin II was used at three doses (100, 400 and 800 µg/ml). For other details see the text. Results for three separate experiments are shown.

cells that contain less than one fasciculata cell/200 glomerulosa cells. However, it is apparent that the low dose of angiotensin II (100 µg) was ineffective in raising cyclic AMP concentrations, yet induced

Fig. 6. Changes in cyclic AMP concentrations in fractionated and unfractionated cells after stimulation with K⁺, 5-hydroxytryptamine, ACTH and angiotensin II

Results are plotted as percentage increase over control values. The histogram records the mean, and the individual results are designated by ● except with 400 µg of [5-isoleucine]-angiotensin/ml (Δ). ■, Unfractionated cells; □, fractionated cells. Concentrations used were: K⁺, 8.4mM; 5-hydroxytryptamine 0.1mM; ACTH, 10m-i.u./ml; [5-valine]angiotensin, 100, 400 or 800 µg/ml. Abbreviation 5-OHTrpNH₂, 5-hydroxytryptamine.

a maximal increase in corticosterone output from the purified glomerulosa cell suspensions.

Discussion

The simple and sensitive saturation assay method for cyclic AMP determinations developed previously (Brown *et al.*, 1971, 1972a) appears to be suitable for the analysis of incubations of isolated adrenal cells. The sensitivity of the assay used is

such that nucleotide content of incubations of 10^5 adrenal cells/ml is easily measured. The low affinity of the binding protein for nucleotides other than cyclic AMP normally eliminates the necessity for extensive sample-purification procedures. This observation has been confirmed by results of determinations of cyclic AMP before and after ion-exchange chromatography both in the present study and in others (Brown *et al.*, 1972a,b). The problem of non-specific interference is discussed in depth by Albano *et al.* (1974b).

A number of agents including 5-hydroxytryptamine, angiotensin, increased K^+ concentration, ACTH and cyclic AMP have been shown previously to stimulate aldosterone production by the adrenal. These studies have been performed on mixed cell populations, whereas only cells of the zona glomerulosa produce aldosterone. However, both zona glomerulosa and zona fasciculata cells produce corticosterone. Therefore interpretation of results with stimuli which affect both zones is difficult. Because of this complication it has not been possible until recently to establish whether ACTH or angiotensin are stimulators of steroidogenesis in the rat zona glomerulosa (Tait *et al.*, 1974).

The pure zona glomerulosa cells used in this study have been shown to be reasonably normal by electron microscopy and by their steroidogenic responses (Tait *et al.*, 1974). Some badly damaged cells present in the original capsular preparation appear to have been eliminated during the fractionation procedure. The cyclic AMP concentrations observed before and after purification were similar. Fasciculata cells have a higher basal concentration of cyclic AMP, so that a decrease in the extent of contamination by fasciculata cells may explain the slight decrease in cyclic AMP concentration after fractionation.

In all experiments, both increased K^+ concentration (from 3.6mM to 8.4mM) and 5-hydroxytryptamine (0.1mM) caused increased cyclic AMP accumulation in both fractionated and unfractionated glomerulosa cells. The increases were approximately similar with these stimuli for both steroid production and cyclic AMP accumulation. That there was not a significant decrease in cyclic AMP or steroid responses after sedimentation would be expected from previous work demonstrating that these stimuli do not affect steroid output from zones other than the zona glomerulosa (Müller, 1965; Kaplan, 1965; Müller & Ziegler, 1968; Haning *et al.*, 1970). This is supported by the observation that neither 5-hydroxytryptamine nor increased K^+ concentration had any significant effect on cyclic AMP concentrations in incubations of cells prepared from decapsulated glands in contrast with the large increases in both cyclic AMP and steroid production induced by ACTH and angiotensin II.

The dose-response relationship of stimulation by K^+ of cyclic AMP and corticosterone production is not easily explicable. An increase in the extracellular K^+ concentrations from 3.6mM to 8.4mM caused both cyclic AMP and corticosterone concentrations to be increased, but within the probable true physiological range (approx. 3.6–6mM- K^+) an interesting phenomenon has been observed in that the consistent increase in corticosterone output was not accompanied by a significant increase in AMP accumulation. Above 8.4mM- K^+ corticosterone production was lower than at 8.4mM- K^+ whereas the cyclic AMP concentration was maintained, suggesting that K^+ exerts a dual effect and causes a decrease in corticosterone that is independent of cyclic AMP concentration. Again, at low K^+ concentrations cyclic AMP accumulation is enhanced, whereas steroid production is not. We have not as yet any firm explanation for this divergence. However, our studies suggest that the action of K^+ at certain doses is accompanied, and perhaps mediated, by an increase in cyclic AMP. To our knowledge the only other reports of effects on cyclic AMP induced by such small alterations in K^+ concentration are those of Saruta *et al.* (1972) using a mixed population of adrenal cells i.e. ox adrenal outer cortical slices and of Boyd *et al.* (1973) on adrenal capsular tissue. The latter workers reported an effect on cyclic AMP concentrations when the extracellular K^+ concentration was increased from 3mM to 6mM. The precise mechanism of the K^+ -induced increases in cyclic AMP concentrations is unknown.

It is clear that 5-hydroxytryptamine at doses of 0.1 μ M to 0.1mM increase cyclic AMP accumulation in zona glomerulosa cells. However, in one experiment the lower dose of 10nM-5-hydroxytryptamine did not increase cyclic AMP accumulation whereas corticosterone output was virtually maximal.

The two other stimuli of steroidogenesis in the zona glomerulosa (ACTH and angiotensin) were also effective in increasing the cyclic AMP concentration in suspensions of both non-purified and purified glomerulosa cells. However, both stimuli exert a considerably greater effect in the zona fasciculata (Tables 2 and 5) and this is reflected in the decrease in the response in terms of cyclic AMP after fractionation of the glomerulosa cells. Nevertheless, there seems little doubt that ACTH and both [5-valine]angiotensin and [5-isoleucine]angiotensin stimulate cyclic AMP accumulation in the purified glomerulosa cells. Equivalent evidence for such an effect on steroid output has been reported by Tait *et al.* (1974). All of the doses of ACTH used in these studies (5 to 40m-i.u./ml) induced an increase in cyclic AMP accumulation. This is, to our knowledge, the first unequivocal demonstration of an effect of ACTH on cyclic AMP concentrations in the zona

glomerulosa. Although angiotensin II at doses above 100 µg/ml induced an increase in cyclic AMP accumulation, the lower dose (100 µg) which caused maximal or near-maximal increase in steroid output had no effect on overall cyclic AMP accumulation. The dose of angiotensin required to give a maximum steroidogenic effect was about fourfold lower for the zona glomerulosa cells compared with the fasciculata cells.

The doses of angiotensin II were high, but [5-isoleucine]angiotensin II had the same effect. Further, the rat adrenal is particularly insensitive to angiotensin. The relative insensitivity of these cell preparations may have been due to the actions of peptidases, since no inhibitors were used. The sensitivity of the cells to K⁺ concentration were as high as in the corresponding experiments *in vivo* (Funder *et al.*, 1969).

The stimulation of cyclic AMP accumulation by angiotensin II in zona fasciculata and to a lesser extent in zona glomerulosa cells may be at variance with other studies. Two reports have indicated that angiotensin II does not stimulate adenylate cyclase of bovine adrenals (Goodfriend & Lin, 1970) or of normal and cancerous rat adrenal tissue (Schorr & Ney, 1971), although binding of angiotensin to bovine adrenal cortical slices has been observed (Lin & Goodfriend, 1970). Saruta *et al.* (1972) did not observe any effect of angiotensin II on outer slices of bovine adrenal glands. However, although this may reflect inter-species differences it is more probable that the considerably lower dose used by these workers may be the main difference.

These results strongly suggest a link between cyclic AMP production and steroidogenesis in the zone of the adrenal gland that specifically secretes aldosterone. All the agents that stimulate the corticosterone (and aldosterone) output of this zone also cause increases in cyclic AMP concentrations, although the dose-response relationships are not always clear. In addition, it has been demonstrated previously that incubation of glomerulosa cells with exogenous cyclic AMP results in increased steroid output (Tait *et al.*, 1974). Nevertheless, these observations raise a general question as to the doses required to elicit increased cyclic AMP concentrations and the associated physiological response. In the present study, as well as in others involving a variety of tissues, including the adrenal cortex, there are degrees of the stimulus which cause a significant physiological response, but at which increases in cyclic AMP concentrations are not observed. Customary explanations advanced for the discrepancy include imprecision of the assay method and sequestration of cyclic AMP in intracellular compartments, implying that influx of cyclic AMP from an inactive to an active pool may be the sole necessary step of the subsequent physiological response. Recent experiments have suggested that

the response in terms of cellular cyclic AMP may be transient (Peytreman *et al.*, 1973; Albano & Brown, 1974) emphasizing the importance of the choice of incubation times particularly when only intracellular cyclic AMP is determined. Clearly more study is required to establish the validity of these or other explanations.

These studies were supported by research grants from the Medical Research Council. We thank Mr. M. S. R. Mee and Mr. D. Atkinson for their technical collaboration.

References

- Albano, J. D. M. & Brown, B. L. (1974) *Biochem. Soc. Trans.* **2**, 412–415
- Albano, J. D. M., Brown, B. L., Ekins, R. P., Price, I., Tait, S. A. S. & Tait, J. F. (1973a) *J. Endocrinol.* **58**, xi
- Albano, J. D. M., Maudsley, D. V., Brown, B. L. & Barnes, G. D. (1973b) *Biochem. Soc. Trans.* **1**, 477–479
- Albano, J. D. M., Brown, B. L., Ekins, R. P., Mee, M., Tait, S. A. S. & Tait, J. P. (1974a) *J. Endocrinol.* in the press
- Albano, J. D. M., Barnes, G. D., Maudsley, D. V., Brown, B. L. & Ekins, R. P. (1974b) *Anal. Biochem.* in the press
- Baumber, J. S., Davis, J. O., Johnson, J. A. & Witty, R. T. (1971) *Amer. J. Physiol.* **220**, 1094–1099
- Beall, R. J. & Sayers, G. (1972) *Arch. Biochem. Biophys.* **148**, 70–76
- Birmingham, M. K., Kurlents, E., Lane, R., Muhlstock, B. & Traikov, H. (1960) *Can. J. Biochem. Physiol.* **38**, 1077–1085
- Boyd, J., Mulrow, P. J., Palmore, W. P. & Silvio, P. (1973) *Circ. Res. Suppl.* **32**, 39–45
- Brown, B. L., Albano, J. D. M., Ekins, R. P., Sgherzi, A. M. & Tempion, W. (1971) *Biochem. J.* **121**, 561–562
- Brown, B. L., Ekins, R. P. & Albano, J. D. M. (1972a) *Advan. Cyclic Nucleotide Res.* **2**, 25–40
- Brown, B. L., Salway, J. G., Albano, J. D. M., Mullin, R. P. & Ekins, R. P. (1972b) *Brit. J. Psychiat.* **120**, 405–408
- Carchman, R. A., Janus, S. D. & Rubin, R. P. (1971) *Mol. Pharmacol.* **7**, 491–498
- Ferguson, J. J. (1963) *J. Biol. Chem.* **238**, 2754–2759
- Funder, J. W., Blair-West, J. R., Coghlan, J. P., Denton, D. A. & Scoggins, B. A. (1969) *Endocrinology* **85**, 381–384
- Goodfriend, T. & Lin, S. Y. (1970) *Circ. Res. Suppl.* **26**, 163–174
- Grahame-Smith, D. G., Butcher, R. W., Ney, R. L. & Sutherland, E. W. (1967) *J. Biol. Chem.* **242**, 5535–5541
- Hanin, R., Tait, S. A. S. & Tait, J. F. (1970) *Endocrinology* **87**, 1147–1167
- Kaplan, N. M. (1965) *J. Clin. Invest.* **44**, 2029–2039
- Karaboyas, G. C. & Koritz, S. B. (1965) *Biochemistry* **4**, 462–468
- Kitabchi, A. E. & Sharma, R. K. (1971) *Endocrinology* **88**, 1109–1116
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Lefkowitz, R. J., Roth, J., Pricer, W. & Pastan, I. (1970) *Proc. Nat. Acad. Sci. U.S.A.* **65**, 745–752

- Lin, S. Y. & Goodfriend, T. L. (1970) *Amer. J. Physiol.* **218**, 1319–1328
- Mackie, C., Richardson, M. C. & Schulster, D. (1972) *FEBS Lett.* **23**, 345–348
- Müller, J. (1965) *Acta Endocrinol. (Copenhagen)* **48**, 283–296
- Müller, J. (1970) *Eur. Clin. Invest.* **1**, 180–187
- Müller, J. & Ziegler, W. H. (1968) *Acta Endocrinol. (Copenhagen)* **59**, 23–35
- Peytreman, A., Nicholson, W. E., Brown, R. D., Liddle, G. W. & Hardman, J. G. (1973) *J. Clin. Invest.* **52**, 835–842
- Saffran, M. & Schally, A. V. (1955) *Endocrinology* **56**, 523–532
- Saruta, T., Cook, R. & Kaplan, N. M. (1972) *J. Clin. Invest.* **51**, 2239–2245
- Sayers, G., Swallow, R. L. & Giordano, N. D. (1971) *Endocrinology* **88**, 1063–1068
- Schorr, I. & Ney, R. L. (1971) *J. Clin. Invest.* **50**, 1295–1302
- Tait, J. F., Tait, S. A. S. & Gould, R. P. (1972) *J. Endocrinol.* **55**, xxxi
- Tait, J. F., Tait, S. A. S., Gould, R. P. & Mee, M. (1974) *Proc. Roy. Soc. Ser. B.* **185**, 375–407
- Tait, S. A. S., Tait, J. F., Okamoto, M. & Flood, C. (1967) *Endocrinology* **81**, 1213–1225
- Tait, S. A. S., Tait, J. F. & Bradley, J. E. S. (1972) *Aust. J. Exp. Biol. Med. Sci.* **50**, 833–846
- Taunton, O. D., Roth, J. & Pastan, I. (1969) *J. Biol. Chem.* **244**, 247–253